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Suitability of calcein as an *in situ* growth marker in burrowing bivalves

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ABSTRACT

The fluorochrome calcein has been used in numerous growth studies of molluscs to internally mark calcified structures. Because of interspecific variations in marking success and possible effects on growth performance, methodological assessments of the suitability of calcein as a growth marker, especially in field contexts, remain necessary. Here we report on the effects of different calcein concentrations (100, 200, 400 and 800 mg l⁻¹) on fluorescent mark deposition, growth rate, density, body condition and size–frequency distribution of an intertidal infaunal bivalve species, *Loripes lacteus* (Linnaeus, 1758), using an outdoor immersion technique. To avoid stress caused by handling and transportation, *in situ* enclosures were placed at seagrass-covered patches during low tide, to which calcein solutions were added. After a 1.3- to 2.6-h period of exposure to calcein, the enclosures were removed. Sites were sampled three months later. All calcein concentrations produced live *L. lacteus* with a clear fluorescent shell mark, but the percentage successfully marked tended to increase with higher calcein concentrations. Furthermore, marking success and growth rate decreased significantly with shell size (i.e., age). Calcein concentration did not measurably affect shell growth rate, body condition and size–frequency distribution, but the numerical densities were lower for sites treated with calcein concentrations ≥ 400 mg l⁻¹. Our results suggest that *in situ* calcein-marking of burrowing lucinid bivalves with low concentrations (100–200 mg l⁻¹) is a non-invasive and rapid method to determine growth rate, provided that the lucinids are not too old.

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1. Introduction

Growth rate is a key parameter in the context of an organism's life history, and therefore research efforts have concentrated on the development of suitable methods to measure growth. Because many bivalve species are commercially important and exploited (McLachlan et al., 1996), growth rates have been well studied in bivalves. Various methods were used to measure rates of increase in shell growth, including size–distribution analysis of single cohorts, analysis of size-increment following mark-and-recapture experiments using different labeling techniques, shell growth-ring analysis, internal growth lines analysis in shell sections, elemental analysis and analysis of stable oxygen isotopes (see Herrmann et al., 2009 and references therein). Size–frequency distribution analyses are widely used to estimate growth rate in temperate species with identifiable cohorts and peaked growth seasons. However, in (sub-) tropical species reproduction and growth often occur throughout the entire year, resulting in the absence of clearly defined cohorts (Sastri, 1979; Urban, 2001). In such species, mark-and-recapture experiments seem more suitable to

estimate growth rates, the more so because they are inexpensive and easy to apply (Fujikura et al., 2003). Yet, traditional marking methods (e.g., surface filing, edge notching, painting labels, tagging) involve the physical handling of the shells and removal from their natural habitat which may lead to disrupted shell growth (Jones et al., 1978). Furthermore, juveniles often cannot be marked due to their small size.

More recently, chemical techniques using fluorochromes (e.g., tetracyclines, xylenol orange, alizarin red and calcein) have been used for growth studies on molluscs (Day et al., 1995; Rowley and MacKinnon, 1995; Kaehler and McQuaid, 1999; Thébault et al., 2006; Riascos et al., 2007; Lucas et al., 2008; Herrmann et al., 2009). The fluorochromes are incorporated into newly mineralized calcium carbonate that makes up the shell, providing a bright green fluorescent reference growth mark that is visible under UV light from which subsequent shell growth can be measured (Wilson et al., 1987). Obviously, to be a suitable growth marker, fluorochromes should be non-toxic and should not adversely affect growth or survivorship. A number of laboratory studies on bivalve molluscs encouragingly indicate that the fluorochrome calcein provides long-lasting fluorescent shell marks without detectable negative effects on growth performance and survivorship (Eads and Layzer, 2002; Heilmayer et al., 2005; Moran and Marko, 2005; Riascos et al., 2007; Lucas et al., 2008). That these laboratory studies indicate calcein to be

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a safe growth marker does not necessarily mean that this will also be the case when used under field conditions (Kaehler and McQuaid, 1999; Thébault et al., 2006; Herrmann et al., 2009), as the toxic effects of a chemical on an organism may differ between field and laboratory (McFarlane and Beamish, 1987).

In the present study we examined the potential of the fluorochrome calcein as an *in situ* growth marker of the thin-shelled burrowing lucinid, *Loripes lacteus* (Mollusca; Bivalvia; Lucinidae), which lives on the seagrass-covered intertidal mudflats of Banc d'Arguin, Mauritania. We tested for the effects of calcein concentration on lucinid marking success, growth rate, density, body condition and size–frequency distribution. As many other deeply burrowed bivalve species (Stanley, 1970), *L. lacteus* is fragile and susceptible to stress by handling. The great advantage of this *in situ* marking method may be that physical stress is minimized because individuals are not touched or moved during the process. An effective non-invasive marker for fragile burrowing bivalves would be a welcome tool for measurements of secondary production in *L. lacteus* and other marine organisms with accretionary growing skeletons.

2. Materials and methods

2.1. Study site

The study was carried out at the intertidal flats close to Iwik, a small fishermen's village in the Banc d'Arguin, Mauritania (19°52.42' N, 16°18.50' W). The marking took place from 25 January to 1 February 2008 and the retrieval of marked bivalves between 24 April and 7 May 2008. The Banc d'Arguin is a major wintering site for migrating shorebirds and is also renowned as a nursery area for fish (Altenburg et al., 1983; Smit and Piersma, 1989; Jager, 1993). The tidal range is 1.5–2 m and the greater part of the intertidal flats is covered with dense seagrass beds, mainly *Zostera noltii* (Wolff and Smit, 1990). Salinity varies between 38 and 42‰, but can reach values of more than 50‰ in sheltered creeks close to the shore (Wolff and Smit, 1990). During the experiment, intertidal flat sediment temperature at 2 cm depth was measured every 15 minutes, with daily averages varying from 17.1 °C (27 January 2008) to 25.4 °C (28 April 2008; Van der Geest et al., unpubl. data). In the seagrass-covered flats of our study area, *Loripes lacteus* is the most abundant bivalve species with an average density of 340–780 individuals per m² (Honkoop et al., 2008; pers. obs.) and maximum densities of 3700 individuals per m² (pers. obs. in April 2007). This lucinid bivalve species lives burrowed in the mud to maximum depths of 10 cm and its thin shells never grow larger than 16 mm (pers. obs.).

2.2. Calcein staining experiment

The experiment was conducted at seven tidal flats distributed around the Iwik peninsula (a 50 km² subsection of the Banc d'Arguin). Within each tidal flat we randomly selected a seagrass-covered site (coordinates of the seven sites were respectively 19°53.615'N, 16°19.619'W; 19°54.996'N, 16°18.881'W; 19°53.554'N, 16°18.822'W; 19°52.244'N, 16°18.210'W; 19°52.588'N, 16°17.260'W; 19°53.647'N, 16°16.561'W; 19°53.035'N, 16°16.370'W). To reach the study sites at low tide, and to work on the usually very muddy tidal flats, we always walked on snow rackets which prevented us from sinking in the mud and from locally damaging the nationally protected mudflats.

During low tide, at each study site, four 15-cm-high PVC rings with a diameter of 30 cm were pushed 10 cm into the sediment (breaking through the seagrass-layer). The PVC rings were placed 2 m apart from each other, in a block design, to keep the environmental conditions at the four “sub-sites” per site as similar as possible. Next, any overlying water enclosed by the PVC rings was removed by gently scooping. To mark the bivalves, the area within each ring was immersed with a 500 ml calcein (Sigma, CAS 1461-15-0) solution of either 100, 200, 400

or 800 mg l⁻¹ (Fig. 1A; solutions were made using 500-μm filtered ambient seawater). These concentrations were selected on the basis of previous studies (Day et al., 1995; Kaehler and McQuaid, 1999; Thébault et al., 2006). At each site, the four calcein concentrations were allocated randomly to each of the four PVC rings. The PVC rings were removed simultaneously per site, always just before being flushed by the incoming tide. As such, there were no differences in calcein immersion time between sub-sites within a site, but only between sites (2.1 ± 0.4 h (mean ± SD). Three months later, a sediment core (internal diameter 15.2 cm) was taken to a depth of 20 cm in the centre of each calcein-immersed sub-site and the content of each core was sieved through a 1-mm sieve and frozen for later analysis. During retrieval of the marked individuals, we also took a 10-cm deep sediment core (internal diameter 20 mm) at each site for analysis of median grain size (MGS) and

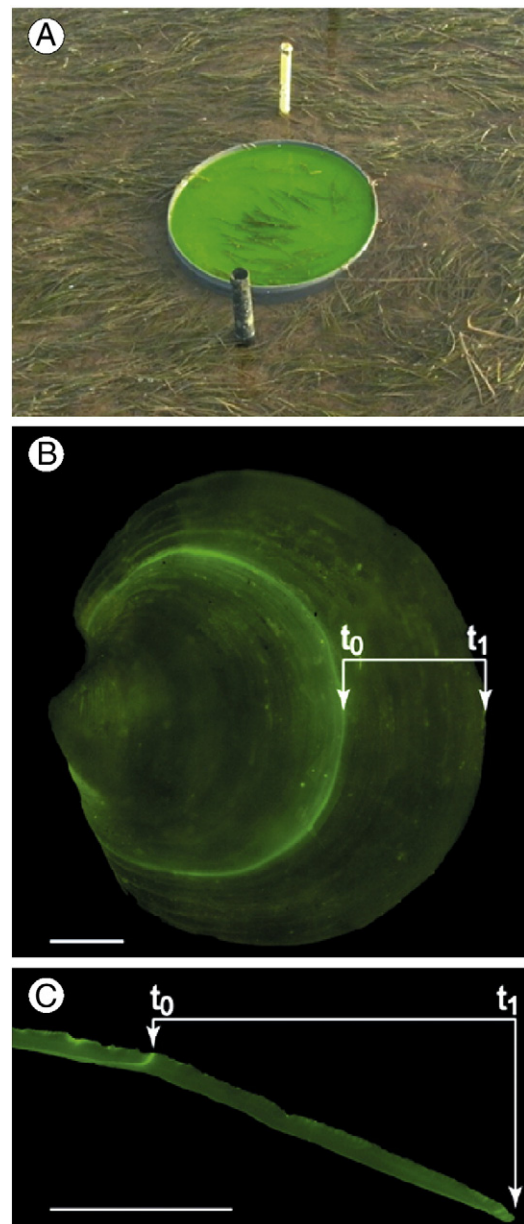


Fig. 1. (A) *In situ* marking of burrowed bivalves by allocation of 500 ml calcein solution of 800 mg l⁻¹ to a seagrass-covered sub-site at a particular site. (B) Photograph of right valve of a calcein marked *Loripes lacteus* shell, viewed with an I2/3 block filter (calcein concentration 800 mg l⁻¹). (C) Photomicrograph of a sagittal section of the left valve of the same calcein marked shell. The position of growing edge at time of calcein administration is given by t_0 ; the position of growing edge three months later is given by t_1 . Thus, t_0 to t_1 represents shell growth (increase in height). Scale bars: 1 mm.

percentage of silt/clay (particles < 63 μm). Additionally, a 10-cm deep seagrass core (internal diameter 70 mm) was taken in order to estimate seagrass abundance (expressed as ash-free dry mass AFDM m^{-2} ; for general methods see Honkoop et al., 2008). The seven sites can be characterized by sediment with MGS of 84.9 μm (range 28.0–160.7), with a percentage of silt/clay of 46.3% (V/V; range 14.2–82.5), a mean total seagrass biomass of 230.9 g AFDM m^{-2} (range 159.2–355.3), of which 82.4 g m^{-2} (range 18.2–162.7) was represented by the leaves and 148.6 g m^{-2} (range 86.4–214.0) by the roots (including rhizomes).

2.3. Sample preparation and detection of calcein marks

Frozen samples were transported to The Netherlands. Shell height was recorded for all individuals in each sample to the nearest 0.1 mm. From each individual, soft tissues were removed and transferred to porcelain crucibles, which were then dried for four days at 60 °C. Dried tissues were weighed and incinerated (560 °C) for 5 hours in order to determine AFDM (Honkoop, 2003). Shells were cleaned in a H_2O_2 -solution (35%) for 24 h to remove the periostracum, rinsed in tap water and dried at room temperature. Before further treatment, shells were stored in the dark to prevent any fluorescence decrease (Wilson et al., 1987). To investigate the presence of a calcein mark at the exterior of the shell, the valves of each shell were observed under an Olympus fluorescence microscope (SZX-12) exciting at 460–490 nm (SZX-RFL3 Cube). A marking quality index (MQI) was defined according to fluorescence intensity and length of the mark: 0 = no mark, 1 = poor mark, 2 = satisfactory mark, 3 = good mark.

2.4. Detection of absolute growth

In general, the internal calcein mark deposited in calcein-treated bivalves is used for growth estimation (Kaehler and McQuaid, 1999; Riascos et al., 2007; Thébault et al., 2006; Herrmann et al., 2009). However, the problem with this procedure is that it is very time-consuming: before the internal calcein mark can be observed, the shell has to be sectioned and polished. Therefore, in order to speed up the measurements of growth estimation, we here aimed to determine growth on the basis of the calcein marks deposited at the exterior of the shell.

By using a micrometer which was installed in the fluorescence binocular, we determined growth in those individuals that displayed a satisfactory or good calcein mark at the exterior of their shell (MQI scores > 1, $N=261$). Growth rate was expressed as the maximum growth axis between the fluorescent calcein mark at the exterior of the shell and the ventral margin to the nearest 0.01 mm divided by the interval (in days) between calcein administration at t_0 and collection of the shell at t_1 (Fig. 1B). Shell height at calcein marking (initial shell height) was expressed as the difference between the final shell height and the shell increase since marking.

In order to validate the assumption that the deposition of the calcein mark at the exterior of the shell can be used to determine growth, we randomly selected 20 marked (MQI > 1) *L. lacteus* individuals of which we also estimated growth by using the internally deposited calcein mark (Fig. 1C). The selected valves were embedded in epoxy resin and after hardening for three days, the valves were sagittally sectioned along the maximum growth axis by using a diamond impregnated blade on an Isomet slow-speed saw machine. The cross-sections were polished on glass slides with different grades of Buehler silicon carbide powder (600–12–5–3 μm), and finally with 0.1 μm Buehler aluminum oxide suspension. The shell slides were observed under a Leitz DM RBE fluorescence microscope (Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany) equipped with an Osram 50-W high-pressure Hg lamp and an I2/3 filter block (excitation filter BP450–490, dichroic mirror RKP510 and emission filter LP515). Growth was determined with a micrometer to the nearest 0.01 mm by measuring the distance between the internal calcein mark at t_0 and the ventral margin at t_1 (Fig. 1C).

A paired t -test revealed no significant differences in growth when either using the external or the internal calcein mark ($t = -0.240$, $df = 18$, $P = 0.81$). Therefore, growth data obtained by using the exterior calcein mark are used for further analysis.

2.5. Statistical analysis

Linear mixed modeling techniques (Pinheiro and Bates, 2000; West et al., 2006; Zuur et al., 2009) were used to assess the effect of the calcein concentration on respectively quality of shell marking (MQI), growth ($\mu\text{m day}^{-1}$), density (in m^{-2} , a net measure of local dispersal, mortality and settlement in the intervening three months), final shell height (a net measure of size-selective mortality and/or migration) and body condition (AFDM corrected for shell height). When applicable, the R^2 value was calculated from the linear mixed model output by using a log-likelihood ratio test (Magee, 1990). Due to non-normality of the MQI-data, we used mean MQI per sub-site. Previous studies have shown that marking quality can be related to shell size (Day et al., 1995; Thébault et al., 2006; Riascos et al., 2007). In order to determine the effect of calcein concentration on mean MQI, we therefore modeled mean MQI as a function of calcein concentration while controlling for mean final shell height per sub-site using the main terms and the two-way interaction (this test could not be done with initial shell height as covariate because initial shell height was only obtained for marked individuals). Additionally, growth rate was modeled as a function of calcein concentration and initial shell height using the main terms and the two-way interaction, as shell growth generally decreases with size (Day et al., 1995; Thébault et al., 2006; Riascos et al., 2007). Both density (m^{-2}) and final shell height (mm) were modeled as a function of calcein concentration. Body mass (AFDM in mg) was modeled as a function of calcein concentration and final shell height using the main terms and the two-way interaction. AFDM and final shell height were log-transformed because mass is scaled exponentially with height.

The term calcein concentration was always fitted as a categorical variable as we expected a threshold concentration at which detrimental effects would occur. All other variables were fitted as continuous variables. Due to the nested structure of the data, site and, where applicable, sub-site (nested in site) were used as random effects. As such, any difference in environmental conditions and calcein immersion time between sites was included in the analysis as part of the random effect of site. Assumptions of normality and homogeneity of residuals were met for all models. Model selection was based on likelihood ratio tests, following a step-down approach

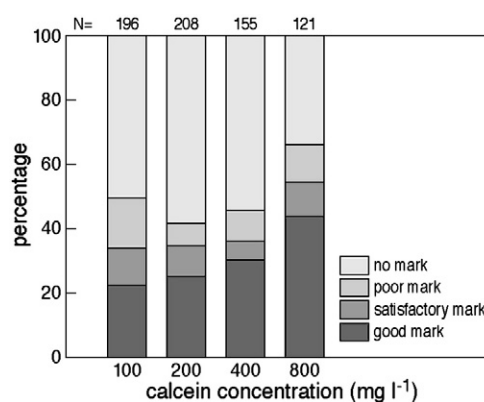


Fig. 2. Plotted as a function of calcein concentration, the percentage of *Loripes lacteus* individuals with respectively no mark (MQI = 0), a poor quality mark (MQI = 1), a satisfactory quality mark (MQI = 2), and a good quality mark (MQI = 3). Sites are pooled.

Table 1

Estimated parameters from the two-way mixed model ANOVA with mean MQI per sub-site as dependent variable, calcein concentration as fixed effect, mean final shell height per sub-site as covariate and site as random effect. We assumed that the random effect of site only acts on the intercept. The random effect representing the between-site variance is 0.13². The estimated value for the residual variance is 0.55².

	Value	SE	df	t-value	P-value
(Intercept)	4.357	1.245	17	3.499	0.003
Mean final shell height (mm)	−0.443	0.167	17	−2.649	0.017
Factor (calcein conc. 200 mg l ^{−1})	0.090	0.297	17	0.302	0.766
Factor (calcein conc. 400 mg l ^{−1})	0.067	0.296	17	0.227	0.823
Factor (calcein conc. 800 mg l ^{−1})	0.583	0.294	17	1.981	0.064

as described in West et al. (2006). All analyses were done in R (R Development Core Team 2008; version 2.10.0) using the package “nlme” (Pinheiro et al., 2008).

At the start of this experiment a parallel experiment on *L. lacteus* was carried out in the same study area in order to test whether the administration of 500 ml of a relatively low concentration of calcein (200 mg l^{−1}) for 2 h would affect density and mean shell height. This parallel study was carried out at 11 paired control and calcein-treated sites, which were sampled in April 2008, three months after adding the calcein solution (just as in the main experiment).

3. Results

In the parallel experiment, a paired *t*-test showed no significant differences in densities between the control and calcein-treated (200 mg l^{−1}) sites (*t* = 0.539, *df* = 10, *P* = 0.60). Additionally, no significant differences in mean shell height were observed between the paired control and calcein-treated sites (*t* = 0.516, *df* = 8, *P* = 0.62). This suggests that application of 500 ml of a concentration of calcein as low as 200 mg l^{−1} does not affect mortality and/or size-selective migration of *L. lacteus*. On the basis of this it seems justified to assume that an even lighter calcein concentration of 100 mg l^{−1}, as used in our main experiment, will have negligible effects on mortality and/or migration of *L. lacteus*.

In the main experiment, calcein produced clearly visible fluorescent marks in shells of *L. lacteus* at all concentrations (Fig. 2). The most parsimonious model for estimating mean MQI contained only the main term mean final shell height and no effect of calcein concentration, with mean MQI increasing for decreasing mean final shell height. This model was significantly better than the intercept-model (*L* = 7.19, *df* = 1, *P* = 0.01). Although not significant, there seems to be a trend that MQI increased for sites that were treated

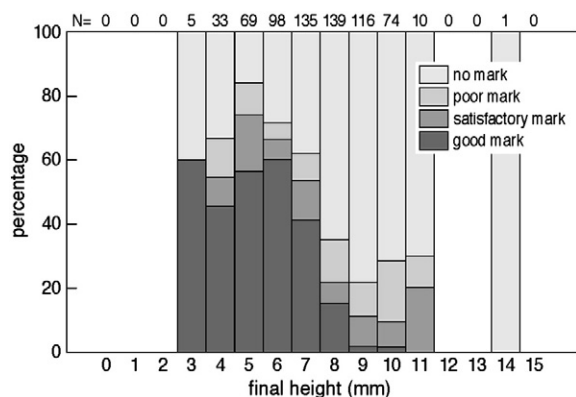


Fig. 3. Plotted as a function of final shell height (mm), the percentage of *Loripes lacteus* individuals with respectively no mark (MQI = 0), a poor quality mark (MQI = 1), a satisfactory quality mark (MQI = 2), and a good quality mark (MQI = 3).

Table 2

Estimated parameters from the two-way mixed model ANOVA with growth rate (μm day^{−1}) of *Loripes lacteus* as dependent variable, calcein concentration as a fixed effect, initial shell height (mm) as a covariate and site and sub-site (nested within site) as random effects. We assumed that the random effect of site only acts on the intercept. The random effect representing the between site variance is 3.91² and the random effect representing the between sub-site variance is 1.89². The estimated value for the residual variance is 7.68².

	Value	SE	df	t-value	P-value
(Intercept)	31.172	2.433	232	12.811	<0.001
Initial shell height (mm)	−1.608	0.345	232	−4.657	<0.001
Factor (calcein conc. 200 mg l ^{−1})	−0.029	1.834	18	−0.016	0.988
Factor (calcein conc. 400 mg l ^{−1})	0.264	1.834	18	0.144	0.887
Factor (calcein conc. 800 mg l ^{−1})	−1.136	1.832	18	−0.620	0.543

with higher calcein concentrations (Fig. 2 and Table 1 for the estimated parameters of the model with the main term calcein concentration still included). That marking success depends on shell size (i.e., age) is also indicated by Fig. 3, which shows that the percentage successfully marked (MQI > 1) is especially high for individuals with a final shell height smaller than 7.5 mm (varying from 53% to 74%), but decreases rapidly for individuals larger than 7.5 mm (varying from 0% to 22%).

Growth rate, controlled for initial shell height, did not differ significantly between calcein concentrations (Table 2). The best model, with only the main term initial shell height, compared with the intercept-model without this term, indicated that the effect of initial shell height on growth rate was highly significant (*L* = 21.41, *df* = 1, *P* < 0.001), although the percent variability explained was low (*R*²_{LR} = 0.08). Individual daily growth rate ranged between 4.7 μm day^{−1} and 56.6 μm day^{−1} and decreased with size (*y* = −1.586 *x* + 30.856, *N* = 261; Fig. 4).

The model with the main term calcein concentration and an intercept-model without this term indicated a nearly significant effect of calcein concentration on density of *L. lacteus* (*L* = 7.24, *df* = 3, *P* = 0.07). This effect is largely due to the drop in densities at sites treated with the highest calcein concentration (800 mg l^{−1}; Fig. 5), which was just significantly lower than at sites treated with the lowest concentration of 100 mg l^{−1} (Table 3; *t* = −2.105, *df* = 18, *P* = 0.049).

There were no differences in size–frequency distribution of *L. lacteus* between calcein treatments, with heights ranging from 3 to 14 mm (Fig. 6). The model with the main term calcein concentration and an intercept-model without this term indeed indicated the

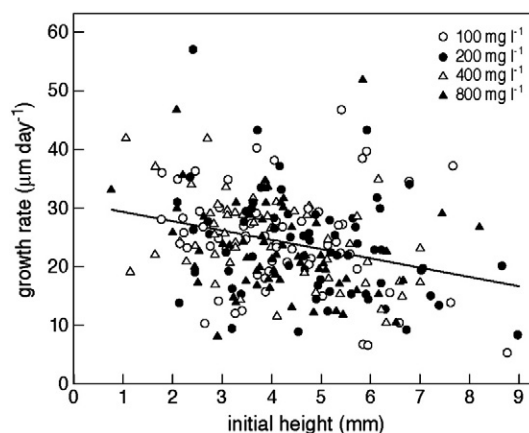


Fig. 4. Relationship between initial shell height (mm) and daily growth rate (μm day^{−1}) of *Loripes lacteus* with the random intercept effect of site removed. Each symbol represents a different calcein concentration. Line represents the significant mixed model with daily growth rate as the dependent variable and initial shell height as the main term in which the non-significant effect of calcein treatment has been removed (*y* = −1.586 *x* + 30.856, *R*²_{LR} = 0.08, *P* < 0.001, *N* = 261).

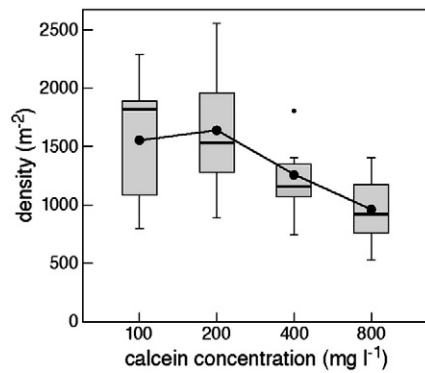


Fig. 5. Density (m^{-2}) of *Loripes lacteus* at various calcein concentrations with the random intercept effect of site removed. Sample size for each treatment is $N=7$. Box-and-whisker plots give the mean (large dot), median (horizontal line inside the box), interquartile range (box), range (bars), and outliers (small dot).

absence of an effect of calcein concentration on final shell height ($L=2.86$, $df=3$, $P=0.41$; site and sub-site (nested within site) were included as random effects in each model).

There was no evidence that body condition ($[\log_{10}(\text{AFDM})]$ controlled for the covariate $[\log_{10}(\text{final height})]$) varied between calcein concentrations (Table 4). Comparison of this model with an intercept-model without the main term calcein concentration, indicated the absence of an effect of calcein concentration on body condition ($L=1.02$, $df=3$, $P=0.80$).

4. Discussion

Immersion of a seagrass-covered patch with a calcein solution ranging from 100 to 800 mg l^{-1} for approximately 2 h was sufficient to produce a bright fluorescent mark on both the in- and outside of shells of *L. lacteus* that live burrowed in muddy seagrass beds. These results corroborate earlier fluorochrome studies demonstrating that immersion in calcein solutions of 100–150 mg l^{-1} were sufficient to mark the shells of gastropods (Moran, 2000; Riascos et al., 2007) and bivalves (Thébault et al., 2006; Riascos et al., 2007; Herrmann et al., 2009). There was a tendency for marking success to increase with higher calcein concentrations (ranging from 34% to 55%; Fig. 2).

Our results suggest an age-dependent effect on the success of calcein marking because small (i.e., young) lucinids were significantly better marked than large ones (Fig. 3 and Table 1). Age-dependent shell marking success has also been observed in abalones (Haliotidae; Day et al., 1995; Riascos et al., 2007), scallops (Pectinidae; Thébault et al., 2006), and surf clams (Mactridae; Riascos et al., 2007). Moreover, growth after marking decreased significantly with shell size (Fig. 4). This inverse relationship reflects a decrease in the rate of shell accretion throughout the life span, an ontogenetic change widely recognized in the life history of many organisms that grow by accretion (e.g., Harrington, 1989). Low growth rates could reduce the transfer of calcein through the mantle epithelium (Day et al., 1995): a 2-h exposure to calcein was probably insufficient to produce an internal fluorescent mark in the larger lucinids.

Table 3

Estimated parameters from the one-way mixed model ANOVA with density (m^{-2}) of *Loripes lacteus* as dependent variable, calcein concentration as fixed effect and site as random effect. We assumed that the random effect of site only acts on the intercept. The random effect representing the between site variance is 701.4². The estimated value for the residual variance is 523.6².

	Value	SE	df	t-value	P-value
(Intercept)	1547.9	330.8	18	4.679	<0.001
Factor (calcein conc. 200 mg l^{-1})	86.4	279.9	18	0.309	0.761
Factor (calcein conc. 400 mg l^{-1})	−330.0	279.9	18	−1.179	0.254
Factor (calcein conc. 800 mg l^{-1})	−589.3	279.9	18	−2.105	0.049

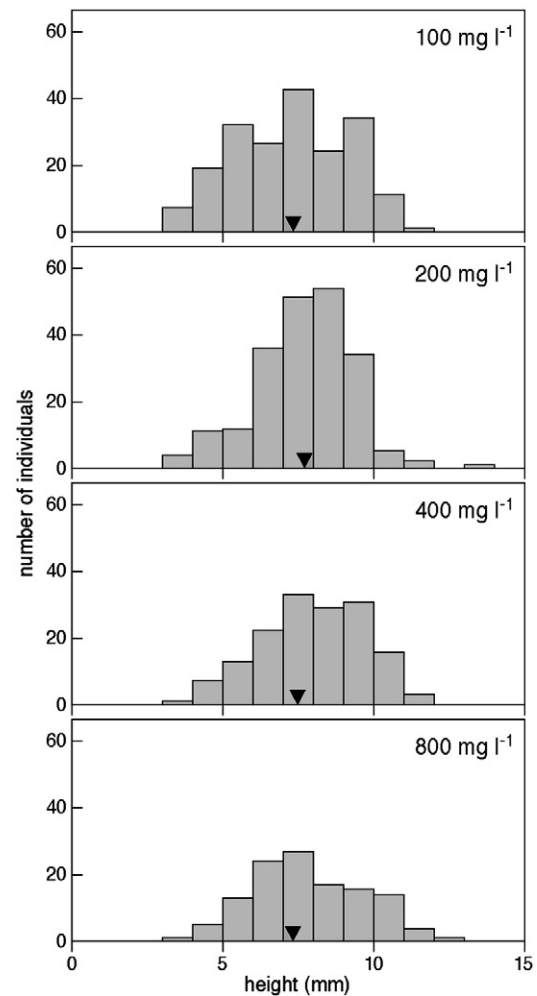


Fig. 6. The size-frequency distribution of *Loripes lacteus* plotted per calcein treatment (100 mg l^{-1} , 200 mg l^{-1} , 400 mg l^{-1} and 800 mg l^{-1}). Sites are pooled. Per calcein treatment, the mean height is indicated by a black arrow.

Nevertheless, there may be other reasons why smaller individuals are more successfully stained. Burrowing depth of *L. lacteus* tends to be a function of size, with larger individuals living more deeply burrowed compared to smaller individuals (pers. obs.). Therefore, one could suggest that being more deeply burrowed, larger lucinids might have less access to the calcein solution allocated, which could result in a decrease in marking success. However, we consider this option as rather unlikely, as even deeply burrowed lucinids have access to the overlying water by means of an anterior inhalant tube, which is used to pump oxygen-rich water to their gills (Allen, 1958; Taylor and Glover, 2000).

Table 4

Estimated parameters from the two-way mixed model ANOVA with log-transformed AFDM in $\text{mg} [\log_{10}(x)]$ as dependent variable, calcein concentration as fixed effect, log-transformed height $[\log_{10}(x)]$ as covariate and site and sub-site (nested within site) as random effects. We assumed that the random effects of site and sub-site only act on the intercept. The random effect representing the between-site variance is 0.000015² and the random effect representing the between sub-site variance is 0.12². The estimated value for the residual variance is 0.15².

	Value	SE	df	t-value	P-value
(Intercept)	−4.315	0.072	646	−60.823	<0.001
Log-transformed height $[\log_{10}(x)]$	2.456	0.059	646	41.353	<0.001
Factor (calcein conc. 200 mg l^{-1})	0.003	0.067	18	0.046	0.964
Factor (calcein conc. 400 mg l^{-1})	0.033	0.067	18	0.492	0.629
Factor (calcein conc. 800 mg l^{-1})	−0.069	0.067	18	−1.023	0.320

In this study, calcein immersion of a 0.071 m² surface area was sufficient to collect marked specimen after an intervening period of three months. The fact that we also collected individuals that were not marked, could partly be explained by local dispersal of unmarked individuals from the surrounding area that moved into the immersed study site after allocation of the calcein solution. To circumvent this problem we suggest the immersion of larger surfaces. Given the relatively low percentage of marked individuals, the method described in this study seems particularly suitable for burrowing bivalves of which natural densities are relatively high, as is the case for *L. lacteus*.

Moran (2000) argues that if inconsistent mark incorporation is observed, then researchers should consider whether excluding unmarked individuals might bias experimental results. In this study, we observed a size-dependent effect on marking success, with less large individuals being marked compared to small ones. If growth rate does not differ within size classes, a size-dependent effect on marking success will not be a constraint to accurately estimate growth for *L. lacteus*, given that growth data are obtained for the full-size range. However, if we assume variation in growth rate between individuals of the same size-class and that slow growing individuals of a certain size-class are less likely to be marked, then this could result in an overestimation of growth for the lucinid population. Indeed, it seems that there is much variation in growth rate within a size-class and the minimum growth rate necessary to be successfully marked is in the order of 8 $\mu\text{m day}^{-1}$ (Fig. 4). As the minimum growth of especially large (i.e., old) individuals could be lower than 8 $\mu\text{m day}^{-1}$, overestimation of growth seems more of a problem for larger individuals. However, even if the observed growth rates for large individuals is overestimated, the fact that production by large individuals is relatively low compared to smaller ones suggests that our marking method seems appropriate if the aim is to estimate secondary production of the entire population of *L. lacteus*.

In our study, the period of calcein immersion was restricted due to the tidal cycle, as immersion took place *in situ* during low tide and lasted until the moment that the study site was flushed by the incoming tide. To avoid major differences in calcein the period of immersion between sites, the time of immersion for all sites was set to the site that was emerged for the shortest period. As a consequence, we used a relatively short immersion time 2.1 ± 0.4 h (mean \pm SD) compared to other fluorochrome studies where minimum immersion times varied from 3 h (Thébault et al., 2006; Riascos et al., 2007; Herrmann et al., 2009) to 12 h (Day et al., 1995). The advantage of a relatively short calcein immersion time is that the period of potential stress to the lucinids is limited. On the contrary, lucinids that are immersed in calcein for a relatively short period, have less time to incorporate the stain into their shell, which could result in a decrease in marking success.

Kaehler and McQuaid (1999) encountered problems when *in situ* immersing mussels in calcein solution as, apart from the growing edge, other parts of the outer calcareous layer were also fluorescently marked probably due to deposition of CaCO₃ by microbial endoliths within the shell, making the identification of a distinct growth mark difficult. In this study, *in situ* immersion of burrowed lucinids in calcein solution resulted in clear well-defined fluorescent growth marks on the exterior of the shell, which might imply that shells of *L. lacteus* are less infested by cyanobacterial shell-borers. This presumed low endolith infestation could be a result of the burrowing life-style of *L. lacteus*, suggesting that this immersion method is especially suitable for marking endobenthic marine organisms.

During immersion, we encountered no problems with calcein solution leaking from the enclosed area, which is likely to be a result of the fine-grained sediment which is typical for the seagrass habitat in our study area. The small grain sizes also prevented the solution from rapid drainage into the sediment. However, when applying this immersion technique in more granule sediments, leaking and rapid drainage of the calcein solution could become more of a problem. In

this case, it is probably better to use a benthic chamber with a closed-circuit flow of the calcein solution as designed by Thébault et al. (2006).

Identification of possible lethal and sub-lethal effects of *in situ* calcein marking is an obvious requirement to assess the suitability of this staining method in bivalve growth studies, especially if growth rate would be slowed down by applying this method. Although not significant, we observed a tendency for *L. lacteus* densities to decrease when applying calcein concentrations ≥ 400 mg l⁻¹ (Fig. 5). As we cannot exclude the possibility that detrimental effects of calcein resulted in increased mortality, care should be taken when applying such high calcein concentrations to mark lucinids. That we observed no detrimental effects of calcein concentration on growth rates agrees with the studies of Rowley and MacKinnon (1995), Moran and Marko (2005) and Riascos et al. (2007). Just as concluded by Riascos et al. (2007) for a gastropod species (*Concholepas concholepas*) and a bivalve species (*Mesodesma donacium*), calcein concentration had no detectable negative effects on body condition in *L. lacteus*. Size-frequency distribution did not significantly differ between calcein treatments (Fig. 6), which suggests that the effect of calcein concentration on local dispersal is limited.

Traditionally, mark-and-recapture techniques used for studying growth were deemed unsuitable for fragile burrowing bivalves as the physical handling and transportation involved would affect growth and survival. That we detected no detrimental effects of our marking method when using calcein concentrations ≤ 200 mg l⁻¹ indicates that *in situ* marking by the fluorochrome calcein provides a suitable growth marker for *L. lacteus* (and potentially other fragile burrowing bivalves). However, as marking success decreased with age, our marking method seems less suitable for bivalve populations that mainly exist of older individuals.

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